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Survival of *Cryptosporidium parvum* Oocysts under Various Environmental Pressures

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The survival of various isolates of *Cryptosporidium parvum* oocysts under a range of environmental pressures including freezing, desiccation, and water treatment processes and in physical environments commonly associated with oocysts such as feces and various water types was monitored. Oocyst viability was assessed by in vitro excystation and by a viability assay based on the exclusion or inclusion of two fluorogenic vital dyes. Although desiccation was found to be lethal, a small proportion of oocysts were able to withstand exposure to temperatures as low as −22°C. The water treatment processes investigated did not affect the survival of oocysts when pH was corrected. However, contact with lime, ferric sulfate, or alum had a significant impact on oocyst survival if the pH was not corrected. Oocysts demonstrated longevity in all water types investigated, including seawater, and when in contact with feces were considered to develop an enhanced impermeability to small molecules which might increase the robustness of the oocysts when exposed to environmental pressures.

The long-acknowledged importance of waterborne transmission of giardiasis has enabled detailed research on the survival of *Giardia* cysts in aquatic environments to be conducted (5). Within the past 10 years, with worldwide recognition of the significance of waterborne cryptosporidiosis, attention has increasingly focused on the viability and survival of *Cryptosporidium* oocysts, and the hardiness of *Cryptosporidium* oocysts and their resistance to commonly utilized disinfection techniques has been widely acknowledged (2). Nevertheless, there remains a paucity of detailed studies on the effects of environmental pressures on the survival of oocysts.

Those studies which have been concerned with the survival of oocysts (1, 2, 4, 6, 8, 9) have tended to focus on the resistance of oocysts to disinfectants. Such studies have been entirely laboratory based and have apparently not taken into consideration the variations in oocysts, both between and within isolates, in their abilities to withstand environmental pressures.

In previous work conducted at the Scottish Parasite Diagnostic Laboratory (SPDL), it was observed that different isolates of oocysts varied in permeability to the fluorescent vital dye 4′,6-diamidino-2-phenylindole (DAPI), and this permeability correlated with viability as defined by in vitro excystation (3). Those oocysts which were not dead but did not include DAPI (DAPI−) were inert at assay but could be converted to viable (DAPI+) oocysts by inclusion of a preincubation trigger. It was suggested that DAPI− oocysts might be more environmentally robust than oocysts whose walls are permeable to DAPI, and data to support this hypothesis were presented.

By monitoring the survival of oocysts from isolates of known initial viabilities under a variety of pressures, the duration and probability of potential threats of infection can be realistically assessed.

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MATERIALS AND METHODS

Sources and purification of oocysts. *C. parvum* oocysts used in this study were obtained from the following sources. Cervine-ovine oocysts (c-o oocysts) were purchased from the Moredun Research Institute (MRI), Edinburgh, Scotland. This strain, originally isolated from deer feces, has been passaged in sheep by MRI. Human oocysts were isolated from fecal samples submitted to SPDL by symptomatic individuals for routine examination. The oocysts were kept as separate isolates. Bovine oocysts were isolated from bovine fecal samples obtained from a study farm by Glasgow Veterinary School or from MRI. Bovine oocysts were also purchased from MRI. This isolate has been passaged in calves by MRI. Oocysts purchased from MRI had been purified by a semiautomated method which involved incubation of the oocysts in 1% sodium dodecyl sulfate and both acid sedimentation and sucrose flotation (15). The oocysts were obtained suspended in phosphate-buffered saline (PBS; pH 7.2) containing 100 U of penicillin and 100 μg of streptomycin per ml.

Oocysts obtained from bovine and human fecal samples were purified at SPDL by ether extraction, sucrose density flotation, and repeated washing and centrifugation steps as described elsewhere (3). Oocysts suspensions were stored at 4°C in reverse osmosis (RO) water, and aliquots were routinely sampled for bacterial and fungal contaminants by culture on blood agar and Sabouraud agar plates.

Assessment of viability. Assessment of oocyst viability was primarily based on the viability assay described by Campbell et al. (3), which is dependent on inclusion or exclusion of two fluorogenic vital dyes, DAPI and propidium iodide (PI), by oocysts. In this assay, oocysts suspended in Hank's balanced salt solution (HBSS) are incubated with DAPI and PI for 2 h at 37°C. Ghost oocysts (nonrefractile empty shells, possibly still containing the residual body) and those oocysts which include PI (PI+) are considered dead. Those oocysts which are neither ghosts nor PI+ are potentially infective. Of these potentially infective oocysts, those which include DAPI (DAPI+) will excyst in a standard 4-h excystation protocol without further triggers. Such oocysts are described as viable. Those oocysts which are potentially infective but

do not include DAPI (DAPI−) can be converted to DAPI+ by a variety of external stimuli or triggers, e.g., preincubation in acidified HBSS. Assessment of viability by inclusion or exclusion of DAPI and PI was checked, except where stated, by a standard 4-h excystation protocol involving incubation at 37°C with freshly made solutions of bovine bile and sodium hydrogen carbonate (3).

Environmental conditions investigated. Six different environmental categories were selected for monitoring oocyst survival. For some categories, oocysts from isolates with different permeabilities were investigated.

(i) Freezing. Aliquots (100 μl) of oocysts (10^6 oocysts per ml of RO water) contained in microcryotubes were either snap-frozen by immersion in liquid nitrogen or slow-frozen by being placed in a freezer set at −22°C. In the latter method of freezing, 19 aliquots of oocysts from the same suspension were frozen periodically over a period of up to 775 h and defrosted simultaneously to assess the effect of freezing over time. Both snap-frozen oocysts and oocysts frozen to −22°C in the freezer were defrosted by thawing at 4°C for between 2 and 3 h. Oocysts were suspended in HBSS, and their viabilities were assessed. The viability of the initial, unfrozen oocyst suspension was also assessed for comparison.

(ii) Desiccation. Aliquots (50 μl) of an oocyst suspension (10^6 oocysts per ml of RO water) were placed on glass slides and air dried at room temperature (between 18 and 20°C). At 2-h intervals for up to 8 h, the oocysts were washed off the slides by immersing the slides in 50-ml tubes containing RO water and agitating the slides. Oocysts were pelleted by centrifugation and suspended in 100 μl of HBSS, and their viabilities were assessed. The viability of the initial oocyst suspension was also assessed for comparison.

(iii) In stools. Stools from three individuals with cryptosporidiosis were stored at 4°C in the dark. At intervals over the course of investigation, a small portion of feces was suspended in 100 μl of HBSS, and the viabilities of the oocysts were assessed by inclusion or exclusion of DAPI and PI. In order to identify oocysts among the contaminating fecal material, oocysts were labeled with a fluorescence-labeled monoclonal antibody (Northumbria Biologicals Ltd., Cramlington, United Kingdom). Correlation between in vitro excystation and fluorogenic vital-dye inclusion or exclusion was not investigated with these three isolates, because contaminating fecal debris in the oocyst suspension made the enumeration of in vitro excystation impractical.

(iv) In laboratory models of water treatment processes. Details (contact times, pH, temperature, etc.) of various water treatment processes, in particular, liming and alum and ferric sulfate flocculating, were obtained from Central Scotland and Grampian Water Boards. Oocyst suspensions (10^6 oocysts per ml of RO water) were subjected to scaled-down laboratory models of these processes. In some instances, the processes were exaggerated (e.g., increasing contact time and/or concentration of the active agent) so that oocysts were exposed to conditions greatly in excess of those they would normally encounter in water treatment. For every treatment investigated (see below), the viabilities of appropriate controls in RO water were assessed in triplicate.

For alum flocculating, oocysts were subjected to (i) Aluminum sulfate (5% aluminum) for 1 h at room temperature, (ii) aluminum sulfate (5% aluminum) corrected to pH 6 with lime (calcium hydroxide) for 1 h at room temperature, (iii) aluminum sulfate (5% aluminum) corrected to pH 6 with sodium hydroxide for 1 h at room temperature, (iv) aluminum sulfate (1.5 ppm, with 0.975 ppm aluminum) for 7 min at room temperature and 4°C, (v) aluminum sulfate (1.5 ppm with 0.975 ppm aluminum) corrected to pH 6 with lime for 7 min at room temperature and 4°C, or (vi) aluminum sulfate (1.5 ppm, with 0.975 ppm aluminum) corrected to pH 6 with lime and the polyelectrolyte Wispofloc N for 7 min at room temperature and 4°C.

For liming and polyelectrolytes, oocysts were subjected to (i) 0.2% lime for 1 h at room temperature, (ii) 0.2% lime corrected to pH 6 with 0.2 M hydrochloric acid for 1 h at room temperature, or (iii) 5% Wispofloc N for 1 h at room temperature.

For ferric flocculating, oocysts were subjected to (i) ferric sulfate (5% iron) for 1 h at room temperature, (ii) ferric sulfate (5% iron) corrected to pH 6 with lime for 1 h at room temperature, (iii) ferric sulfate (5% iron) corrected to pH 6 with sodium hydroxide for 1 h at room temperature, (iv) ferric sulfate (3.5 ppm, with 16 ppm iron) for 1 and 5 h at room temperature and 4°C, or (v) ferric sulfate (3.5 ppm, with 16 ppm iron) corrected to pHs 6 and 9 with lime for 1 and 5 h at room temperature and 4°C.

(v) In semipermeable containers in selected environments. A semipermeable container was developed to allow oocysts to be in contact with defined environments and to allow regular sampling with minimal risk of bacterial contamination (10). The container was accessed through silicon bungs (Neville and Moore Ltd., Southwater, United Kingdom) which self-seal on puncture with a hypodermic needle. Contents of the container were in contact with the environment through a nylon-6 semipermeable membrane (Vokes Ltd., Guildford, United Kingdom) with a nominal porosity of 0.2 μm. Before oocysts were injected into these containers, the containers were thoroughly sterilized in a 1% hypochlorite solution which was then washed out by repeated immersion of the containers in RO water. Oocyst suspensions were treated overnight with an antibiotic solution before being injected into the containers. The antibiotic solution (4 μl of gentamicin [40 μg/μl], 20 μl of flucytosine [10 μg/μl], and 50 μl of penicillin G [1 μg/μl] per ml of oocyst suspension) did not affect the viability of any oocyst suspensions. Approximately 2.8×10^7 oocysts were injected into each container, and the containers were then placed in defined environments, with two containers per environment. Control oocysts were injected into containers which were stored in 4 liters of RO water at 4°C in the dark. The RO water was changed at fortnightly intervals. Three environments of interest in terms of how they would affect the viability of *Cryptosporidium* oocysts over time were selected.

For the tap water environment, a light-proof tank was plumbed into the main water supply, with water entering at the bottom of the tank and flowing out at the top. This allowed a constant throughput of tap water from water mains to be maintained.

For the river water environment, containers of oocysts were placed in a 4-liter plastic jar, each side of which had 10 holes (20-mm diameter each) drilled into it. The jar was weighted and submerged in a river. Retrieval of the oocyst containers from the jar was facilitated by tethering the jar to a tree. No attempt was made to measure physiochemical parameters within the river.

For the cow feces environment, approximately 25 liters of semisolid cow feces was obtained from Glasgow Veterinary School. The feces were stored in a plastic bucket in the dark in an outside storage area. The containers of *Cryptosporidium* oocysts were buried in the feces, with retrieval being facilitated by placing the containers in a cage of tensile

TABLE 1. Effect of freezing on survival of *Cryptosporidium* oocysts from c-o isolate with high initial DAPI+/DAPI− ratio

Time (h)	% Oocysts		
	Dead	DAPI+	DAPI−
0	21.0	75.0	4.0
21	67.0	26.0	7.0
114	79.4	16.8	3.8
152	92.1	6.9	1.0
297	89.1	10.9	0.0
436	97.1	2.9	0.0
532	97.0	3.0	0.0
633	98.1	1.9	0.0
775	98.2	1.8	0.0

stainless-steel wire. An electronic temperature probe was also attached to the cage.

The oocysts, including controls, were sampled at approximately fortnightly intervals. The containers were retrieved from their environments and shaken vigorously to ensure thorough mixing and to reduce adhesion of oocysts to the container walls. Approximately 0.25 ml of oocyst suspension was taken from each container by hypodermic syringe through the self-sealing silicon bungs. The samples were kept as close to 4°C as possible until all samples had been collected. No longer than 5 h after collection, the samples were suspended in 100 μl of HBSS, and their viabilities were assessed.

(vi) Seawater. As the semipermeable containers were not considered robust enough to survive immersion directly in the sea, the survival of oocysts in seawater was investigated in the laboratory. Suspensions of oocysts (10^6 oocysts per ml of RO water) were suspended in 1 ml of seawater collected from the Ayrshire coast, and then each suspension was divided into six separate aliquots in microcryotubes and stored at 4°C in the dark. At recorded time intervals of up to 35 days, an aliquot of each isolate was suspended in HBSS, and the viability was assessed. The viability of the initial oocyst suspension was also assessed for comparison.

Statistical analyses. In order to assess the viability of the oocyst population, 100 oocysts were counted at each assay. Triplicate assays of control oocysts were done. Chi-square tests, Mann-Whitney U tests, and calculation of correlation coefficients were performed where appropriate by using a MINITAB statistics package.

RESULTS

Correlation of viability assessed by in vitro excystation and inclusion or exclusion of DAPI and PI. For all environmental pressures investigated, apart from survival of oocysts in human stools, viability as assessed by inclusion or exclusion of DAPI and PI was confirmed by performing 4-h in vitro excystations on randomly selected samples. Correlation was excellent, with a correlation coefficient for the combined results, both control and experimental, of 0.991.

Freezing. Snap-freezing of oocysts resulted in 100% death. Slow freezing, however, was less effective at killing oocysts (Table 1). After 21 h at −22°C, only 67% of oocysts had been killed, and even though this proportion increased to over 90% after 152 h, a small proportion of oocysts were still viable even after 750 h.

Desiccation. Although after only 2 h of air drying at room temperature 3% of the oocysts were still viable, slightly longer periods of drying resulted in 100% death of the oocysts (Table 2).

In stools. Variation in the proportions of dead (PI+ and ghost), DAPI+, and DAPI− oocysts between the three isolates of *Cryptosporidium* oocysts were considerable (Table 3). However, each isolate followed a similar pattern over time, with a gradual but perceptible increase in the proportion of dead oocysts over the first 48 days of the investigation and corresponding fluctuations in the proportions of DAPI+ and DAPI− oocysts. However, by the next sampling point 130 days later, the proportion of dead oocysts had increased considerably, especially in stools A and C (Table 3).

In laboratory models of water treatment processes. (i) Alum floccing. Although contact with high concentrations of aluminum for prolonged periods caused a significant increase in oocyst death (Table 4; $P < 0.001$), alum floccing at the pH, concentration, and contact time utilized by the water industry appeared to have no impact on the viability of *Cryptosporidium* oocysts (Tables 5 and 6). Likewise, oocyst viability was not affected, even with high aluminum concentrations and prolonged contact times, if pH was corrected

TABLE 2. Effect of drying on survival of *Cryptosporidium* oocysts from c-o isolate with high initial DAPI+/DAPI− ratio

Time (h)	% Oocysts		
	Dead	DAPI+	DAPI−
0	23.7	72.8	3.5
2	97.0	3.0	0.0
4	100.0	0.0	0.0
6	100.0	0.0	0.0
8	100.0	0.0	0.0

TABLE 3. Survival of *Cryptosporidium* oocysts in stools from patients with cryptosporidiosis

Stool and type of oocyst	% Oocysts on day:							
	Initial	1	5	12	20	34	48	178
A								
Dead	2.0	2.0	3.0	7.0	5.7	13.0	17.0	93.0
DAPI+	2.0	6.0	10.0	10.0	6.7	10.0	9.0	6.0
DAPI−	96.0	92.0	87.0	83.0	87.6	77.0	74.0	1.0
B								
Dead	20.0	19.8	18.0	18.0	24.0	28.2	28.0	41.0
DAPI+	0.0	16.7	20.0	17.0	8.7	7.8	4.0	24.0
DAPI−	80.0	63.5	52.0	65.0	67.3	64.0	68.0	35.0
C								
Dead	50.0	63.3	63.2	64.0	60.5	62.3	66.0	100.0
DAPI+	25.5	20.9	23.1	25.0	25.7	22.8	24.0	0.0
DAPI−	24.5	15.8	13.7	11.0	13.8	14.9	10.0	0.0

TABLE 4. Effect of 5% aluminum (aluminum sulfate) or 5% Wispofloc N (polyelectrolyte) on viability of *Cryptosporidium* oocysts from c-o isolate with high initial DAPI+/DAPI− ratio

Treatment[a]	% Oocysts		
	Dead	DAPI+	DAPI−
5% Al	42.3	54.1	3.6
5% WfN	22.3	72.8	4.9
Control (mean + SD, $n = 3$)	20.1 ± 2.7	75.4 ± 2.6	4.5 ± 1.1

[a] Treatments were for 1 h at room temperature. WfN, Wispofloc N.

TABLE 5. Effect of aluminum (aluminum sulfate) on viability of two isolates of *Cryptosporidium* oocysts

Isolate and condition[a]	% Oocysts (mean + SD)[b]		
	Dead	DAPI+	DAPI−
A[c] at room temp			
Control	14.9 ± 3.6	77.0 ± 4.5	8.1 ± 6.2
1.5 ppm Al	18.3 ± 4.1	75.9 ± 5.6	5.8 ± 2.6
1.0 ppm Al	23.6 ± 6.8	70.6 ± 5.4	5.8 ± 1.6
A at 4°C			
Control	20.0 ± 7.1	72.5 ± 7.5	7.5 ± 3.9
1.5 ppm Al	22.0 ± 5.3	73.5 ± 4.2	4.5 ± 2.3
1.0 ppm Al	24.0 ± 5.9	71.8 ± 5.2	4.2 ± 1.1
B[d] at room temp			
Control	16.2 ± 2.7	52.1 ± 4.6	31.6 ± 6.1
1.5 ppm Al	18.9 ± 6.1	58.4 ± 6.3	22.7 ± 1.0
1.0 ppm Al	16.3 ± 2.3	60.3 ± 5.3	23.4 ± 2.1
B at 4°C			
Control	15.8 ± 2.0	52.9 ± 5.7	31.3 ± 5.1
1.5 ppm Al	15.9 ± 5.4	56.2 ± 5.7	27.8 ± 5.4
1.0 ppm Al	22.5 ± 6.0	52.3 ± 4.0	25.2 ± 3.0

[a] All treatments were for 7 min.
[b] $n = 3$.
[c] C-o isolate with high initial DAPI+/DAPI− ratio.
[d] Bovine isolate (SPDL purified) with low initial DAPI+/DAPI− ratio.

from approximately 1.5 to approximately 6 by addition of either sodium hydroxide or lime (Table 7).

(ii) Liming and polyelectrolytes. High concentrations of lime over prolonged periods affected the viabilities of *Cryptosporidium* oocysts (Table 8), with a significant increase in the proportion of dead oocysts ($P < 0.01$) and corresponding decreases in the proportions of DAPI+ and DAPI− oocysts. However, if the lime was pH corrected from approximatley 10.5 to approximately 6 by the addition of 1% hydrochloric acid, viabilities of oocysts were not altered (Table 7).

High concentrations of the polyelectrolyte Wispofloc N (pH 6 without correction) did not affect viabilities of oocysts (Table 6).

TABLE 6. Effect of aluminum (aluminum sulfate) on viability of *Cryptosporidium* oocysts from c-o isolate with high initial DAPI+/DAPI− ratio

Temp and treatment[a]	% Oocysts		
	Dead	DAPI+	DAPI−
Room temp			
Control	15.0	81.0	4.0
Control (pH 6)	21.4	72.3	6.3
Control + WfN	14.0	82.0	4.0
Control (pH 6) + WfN	17.6	77.8	4.6
0.975 ppm Al (pH 6)	21.0	76.0	3.0
0.975 ppm Al (pH 6) + WfN	22.5	71.6	5.9
1.5 ppm Al (pH 6)	21.0	75.0	4.0
1.5 ppm Al (pH 6) + WfN	19.8	74.3	5.9
4°C			
Control	17.8	77.2	5.0
Control (pH 6)	20.0	72.3	7.7
Control + WfN	15.4	79.8	4.8
Control (pH 6) + WfN	17.6	77.5	4.9
0.975 ppm Al (pH 6)	18.0	75.0	7.0
0.975 ppm Al (pH 6) + WfN	22.6	70.8	6.6
1.5 ppm Al (pH 6)	22.0	74.0	4.0
1.5 ppm Al (pH 6) + WfN	21.9	74.9	4.2

[a] Aluminum was corrected to pH 6 with lime. All treatments were for 7 min. WfN, Wispofloc N.

TABLE 7. Effects of pH-corrected aluminum (5% aluminum sulfate) and pH-corrected lime (0.2% calcium hydroxide) on viability of *Cryptosporidium* oocysts from bovine isolate (SPDL purified) with low initial DAPI+/DAPI− ratio

Treatment[a]	% Oocysts		
	Dead	DAPI+	DAPI−
Control (mean ± SD, $n = 3$)	28.5 ± 4.6	40.4 ± 1.6	31.1 ± 4.2
5% Al[b]	23.6	41.5	34.9
5% Al[c]	27.2	39.8	33.0
Lime[d]	32.0	41.0	27.0

[a] All treatments were for 1 h at room temperature.
[b] Corrected to pH 6 with lime.
[c] Corrected to pH 6 with NaOH.
[d] Corrected to pH 6 with HCl.

(iii) Ferric sulfate flocing. High concentrations of ferric ions resulted in a significant increase in death of oocysts (Table 9; $P < 0.001$). However, when pH was corrected from approximately 1.5 to approximately 6 by addition of either sodium hydroxide or lime, no impact on viabilities of *Cryptosporidium* oocysts was detected (Table 9).

Lower concentrations of ferric ions affected viability of oocysts at 4°C only if contact time with 16 ppm Fe^{3+} was prolonged for 5 h and pH was raised to pH 9 (Table 10; $P < 0.05$). At room temperature, 16 ppm Fe^{3+} at pH 9 killed a significant proportion of oocysts ($P < 0.05$) if the contact time was reduced to 1 h. If contact time was prolonged to 5 h, 3.5 and 16 ppm Fe^{3+} both without pH correction and corrected to pH 9 significantly increased death of oocysts ($P < 0.001$).

In semipermeable containers in selected environments. In all environments, the proportion of dead oocysts increased gradually over time (Table 11). The proportion of dead oocysts was significantly higher ($P < 0.05$) at final sampling than initially for all environments and for both isolates tested. Death rates did not differ significantly between isolates, and for both isolates, the most noticeable impact on oocyst survival was observed in those oocysts in contact with either river or tap water. Observations of the alterations in proportions of DAPI+ and DAPI− oocysts of the two isolates in the different environments were interesting (Table 11). In the c-o isolate (high DAPI+/DAPI− ratio initially) the proportion of DAPI+ oocysts decreased steadily over the period of investigation in all environments.

However, in the bovine isolate (low DAPI+/DAPI− ratio initially), a continuous reduction in the proportion of DAPI+ oocysts over the period of investigation was observed only in the oocyst suspension held in cow feces. In the other environments, a different pattern was observed, with an initial increase in the proportion of DAPI+ oocysts. In suspensions of the bovine isolate of oocysts in contact with

TABLE 8. Effect of lime (0.2% calcium hydroxide) on viability of *Cryptosporidium* oocysts from bovine isolate (SPDL purified) with low initial DAPI+/DAPI− ratio

Treatment[a]	% Oocysts		
	Dead	DAPI+	DAPI−
Control (mean ± SD, $n = 3$)	28.0 ± 0.9	42.1 ± 1.2	29.9 ± 2.0
Lime	49.5	25.7	24.8

[a] All treatments were for 1 h at room temperature.

TABLE 9. Effect of 5% iron (ferric sulfate) on viability of *Cryptosporidium* oocysts from c-o isolate with high initial DAPI+/DAPI− ratio

Treatment[a]	% Oocysts		
	Dead	DAPI+	DAPI−
Control (mean ± SD, $n = 3$)	27.8 ± 3.5	70.2 ± 2.1	2.0 ± 3.4
5% Fe	97.0	3.0	0.0
5% Fe[b]	26.3	68.4	5.3
5% Fe[c]	31.4	64.4	4.2

[a] Treatments were for 1 h at room temperature.
[b] Corrected to pH 6 with lime.
[c] Corrected to pH 6 with NaOH.

both tap and river water, the proportion of DAPI+ oocysts reached a peak after 11 days. These levels were significantly higher than the original levels ($P < 0.01$).

For both isolates held in aqueous environments, the proportion of DAPI− oocysts fell over the period of investigation (Table 11). However, for the suspensions of oocysts held in cow feces, an initial increase in the proportion of DAPI− oocysts was observed, peaking after 33 days. At the next sampling point (over 140 days later), the proportion of DAPI− oocysts had declined again in cow feces isolates but was still significantly higher than initially ($P < 0.05$) in the c-o isolate.

Monitoring of the temperature in the cow feces revealed no significant fluctuations in temperature.

In seawater. For both c-o and human isolates of *Cryptosporidium* oocysts, the proportion of dead oocysts increased over the 35-day period of investigation (Table 12), and the proportion of dead oocysts was significantly higher ($P < 0.001$) at the final sampling than initially. No difference in survival of the isolates was detected.

Differences between isolates were, however, observed when alterations in the proportions of DAPI+ and DAPI− oocysts were considered (Table 12). In the c-o isolate, the proportions of both DAPI+ and DAPI− oocysts gradually decreased over the 35-day period, and the proportion of DAPI+ oocysts observed at the final sampling was significantly reduced compared with that of the initial sample ($P < 0.001$). In the human isolate, while the proportion of DAPI− oocysts diminished over the 35-day period, being significantly reduced at the final sampling compared with the original levels ($P < 0.001$), the proportion of viable (DAPI+) oocysts increased, and at the final sampling, their proportion was significantly higher than the initial level ($P < 0.001$).

TABLE 10. Effect of iron (ferric sulfate) on viability of *Cryptosporidium* oocysts from c-o isolate with high initial DAPI+/DAPI− ratio)

Conditions and treatment[a]	% Oocysts		
	Dead	DAPI+	DAPI−
Room temp, 1 h			
Control (mean ± SD, $n = 3$)	32.5 ± 7.2	65.7 ± 7.3	1.9 ± 0.9
3.5 ppm Fe	31.4	63.8	4.8
3.5 ppm Fe (pH 6)	39.0	58.2	2.8
3.5 ppm Fe (pH 9)	41.4	57.7	0.9
16 ppm Fe	45.2	50.9	4.0
16 ppm Fe (pH 6)	37.6	59.6	2.8
16 ppm Fe (pH 9)	50.0	48.2	1.8
Room temp, 5 h			
Control (mean ± SD, $n = 3$)	32.0 ± 4.4	65.2 ± 7.9	2.7 ± 2.4
3.5 ppm Fe	58.4	36.6	5.0
3.5 ppm Fe (pH 6)	38.0	57.0	5.0
3.5 ppm Fe (pH 9)	76.2	21.9	1.9
16 ppm Fe	49.6	46.1	4.3
16 ppm Fe (pH 6)	40.5	57.0	2.5
16 ppm Fe (pH 9)	56.9	37.6	5.5
4°C, 1 h			
Control (mean ± SD, $n = 3$)	36.9 ± 3.0	60.1 ± 3.1	2.9 ± 2.0
3.5 ppm Fe	44.7	51.8	3.5
3.5 ppm Fe (pH 6)	34.0	63.1	2.9
3.5 ppm Fe (pH 9)	53.8	42.3	3.9
16 ppm Fe	43.6	54.5	1.9
16 ppm Fe (pH 6)	39.8	59.2	1.0
16 ppm Fe (pH 9)	41.0	56.2	2.8
4°C, 5 h			
Control (mean ± SD, $n = 3$)	42.5 ± 4.3	54.4 ± 2.4	3.1 ± 1.3
3.5 ppm Fe	46.9	49.6	3.5
3.5 ppm Fe (pH 6)	36.9	51.2	2.9
3.5 ppm Fe (pH 9)	52.6	44.0	3.4
16 ppm Fe	52.5	44.6	2.9
16 ppm Fe (pH 6)	43.4	53.3	3.3
16 ppm Fe (pH 9)	57.4	38.6	4.0

[a] Lime was used to correct pHs.

TABLE 11. Effect of contact with various environments on two isolates of *Cryptosporidium* oocysts

Isolate and type of oocyst	% Oocysts on day:					
	0	11	19	33	47	176
C-o[a]						
Control (RO water)						
Dead	22.3	24.8	26.3	25.7	28.5	66.7
DAPI+	74.4	71.3	69.0	69.5	68.6	31.5
DAPI−	3.3	3.9	4.7	4.8	2.9	1.8
Tap water						
Dead	22.3	25.0	22.5	30.3	35.6	96.0
DAPI+	74.4	70.0	72.1	69.6	63.9	3.0
DAPI−	3.3	5.0	5.4	0.1	0.5	1.0
River water						
Dead	22.3	24.6	33.0	44.3	57.1	89.0
DAPI+	74.4	71.6	62.0	49.9	50.2	10.0
DAPI−	3.3	3.8	5.0	5.8	2.7	1.0
Cow feces						
Dead	22.3	26.7	30.8	33.0	38.3	72.0
DAPI+	74.4	63.8	45.8	21.5	16.8	14.0
DAPI−	3.3	9.5	23.4	45.5	44.9	14.0
Bovine[b]						
Control (RO water)						
Dead	16.2	19.1	17.7	28.3	24.0	71.2
DAPI+	44.6	49.5	46.9	53.8	65.5	24.9
DAPI−	39.2	31.4	35.4	17.9	10.5	3.9
Tap water						
Dead	16.2	28.1	36.2	34.6	39.0	99.0
DAPI+	44.6	56.3	50.2	52.6	58.0	1.0
DAPI−	39.2	15.6	13.6	12.8	3.0	0.0
River water						
Dead	16.2	25.0	18.9	40.0	54.9	99.0
DAPI+	44.6	55.0	44.1	31.0	30.4	1.0
DAPI−	39.2	20.0	37.0	29.0	14.7	0.0
Cow feces						
Dead	16.2	17.0	16.5	22.8	33.0	60.4
DAPI+	44.6	41.1	28.4	13.4	12.0	18.8
DAPI−	39.2	41.9	55.1	63.8	55.0	20.8

[a] High initial DAPI+/DAPI− ratio.
[b] Low initial DAPI+/DAPI− ratio.

TABLE 12. Viability of two isolates of *Cryptosporidium* oocysts during immersion in seawater

Isolate and type of oocyst	% Oocysts on day (mean ± SD)[a]:						
	0	2	7	14	21	28	35
C-o[b]							
Dead	18.0 ± 2.0	25.3 ± 11.0	31.3 ± 2.5	31.7 ± 5.7	34.0 ± 2.0	33.7 ± 5.0	43.7 ± 3.2
DAPI+	77.0 ± 2.5	70.0 ± 10.0	64.7 ± 3.8	65.0 ± 6.6	64.0 ± 3.5	64.7 ± 5.0	55.6 ± 4.1
DAPI−	4.3 ± 2.5	4.0 ± 0.0	4.0 ± 2.0	3.3 ± 1.2	2.0 ± 0.2	1.7 ± 0.2	0.7 ± 0.2
Human[c]							
Dead	14.0 ± 1.0	28.0 ± 6.0	22.0 ± 2.0	26.7 ± 6.1	28.0 ± 4.0	29.3 ± 6.8	32.7 ± 3.6
DAPI+	7.3 ± 0.6	34.0 ± 5.3	53.3 ± 5.1	55.0 ± 5.7	56.0 ± 2.0	52.0 ± 1.0	56.4 ± 2.8
DAPI−	78.7 ± 1.5	38.0 ± 2.0	24.7 ± 4.0	18.7 ± 3.2	16.0 ± 2.0	18.0 ± 0.8	12.9 ± 2.0

[a] $n = 3$
[b] High initial DAPI+/DAPI− ratio.
[c] Low initial DAPI+/DAPI− ratio.

DISCUSSION

The robust nature of coccidian oocysts has long been recognized. They are well known to be resistant to many forms of environmental stress that would prove lethal to other species of infectious agents. Desiccation and freezing have apparently been the exceptions (2). However, although data presented here confirm that desiccation is lethal, we have also demonstrated that a small proportion of oocysts are capable of surviving for at least 775 h at −22°C. Current knowledge on the infectious dose of oocysts is limited, and therefore we feel unable to state whether or not infectivity would be retained in this instance. Other research (13) has demonstrated that a number of cryopreservative techniques (including freezing to −20°C for 14 days in PBS followed by a slow thaw) apparently destroy the infectivity of *Cryptosporidium* oocysts for mice. Tzipori (14) also claimed that oocyst infectivity was destroyed by exposure to temperatures below freezing, but no data were provided to support this. Nevertheless, an anecdotal report (7) describes infection with *Cryptosporidium* spp. apparently as a result of accidental ingestion of frozen tripe contaminated with oocysts, and more-recent work (12) describes a technique for cryopreservation of *Cryptosporidium* oocysts in 10% dimethyl sulfoxide. Our data also suggest that it may be unwise to assume that *Cryptosporidium* oocysts that have been frozen are incapable of infection.

Our investigation in laboratory models of the effects of water treatment processes on the viability of *Cryptosporidium* oocysts suggests that although contact with lime, ferric sulfate, and alum individually can kill oocysts, this is a phenomenon of alkalinity (lime) or acidity (alum and ferric sulfate). If pH, contact time, and concentrations are corrected to those at which processes are normally implemented at water treatment plants, these treatments appears to have no significant impact on viability of *Cryptosporidium* oocysts.

Results from monitoring viabilities of oocysts in various environments reemphasize the resistance of the oocysts to the pressures they are likely to encounter in a nonlaboratory setting. However, a significant proportion of oocysts were killed in all environments over the 6-month period of investigation. Previous research (3) suggested that oocysts which were impermeable to DAPI (DAPI−) might be more robust than those which were permeable to DAPI (DAPI+). Furthermore, research has also demonstrated that permeability of oocysts to DAPI is a dynamic state which may be either decreased or increased by selected treatments (11). Although no significant difference in survival between the isolate with a high proportion of DAPI+ oocysts and the isolate with a high proportion of DAPI− oocysts was found, it appeared that in the aqueous environments investigated, the DAPI− oocysts were transformed to DAPI+ oocysts. These DAPI+ oocysts might then succumb to environmental pressures and die. Thus, although in this study no difference in survival of the two isolates was detected, the transformation of DAPI− oocysts to DAPI+ oocysts suggests that in some circumstances, this impermeability may confer some protection. One hour of exposure to 0.1 M sodium hydroxide is one example of this "short, sharp shock" treatment whose impact is more lethal to DAPI+ oocysts than to DAPI− oocysts (3), but more-prolonged exposure to such alkaline solutions would probably be lethal to all oocysts.

Similar information was collected for suspensions of oocysts in seawater. Although there was a significant increase in the percentage of dead oocysts, a large proportion survived. The diluting effect of the sea is enormous, but the risk of contracting cryptosporidiosis from accidentally ingesting oocysts while swimming in coastal areas contaminated with sewage cannot be dismissed. Again, no difference in survival of the two isolates was detected, but it was observed that in the human isolate, there was an increase in the proportion of oocysts permeable to DAPI.

Both isolates of oocysts stored in cow feces demonstrated an interesting decrease in permeability to DAPI over the first 33 days of the study, and the proportion of oocysts still surviving at the end of the study was not significantly different from that observed in controls in RO water. These results suggest that a component of feces, possibly a mucopolysaccharide, might insert into the oocyst wall, decreasing its permeability to DAPI. This fecal component, possibly affording the oocyst further protection from environmental stresses, might be easily lost in digestive processes so that oocysts reaching the intestine would be capable of excystation and infection. In stool samples from patients with symptomatic cryptosporidiosis, a relatively high proportion of oocysts were impermeable to DAPI initially, but this percentage decreased over the period of investigation.

From interpretation of these data, three major points can be concluded. (i) Desiccation of *Cryptosporidium* oocysts appears to be 100% lethal, but a small proportion are capable of surviving long periods of being frozen. Further work should elucidate the risk of infection from such a small proportion of oocysts, but until such work is completed, neither frozen food nor ice should be considered without risk in the transmission of oocysts of *C. parvum*. (ii) The water treatment processes described here have negligible effect on

the survival of *Cryptosporidium* oocysts, although both high and low pH have a significant impact on oocyst viability. (iii) Isolates of oocysts which contained a large proportion of oocysts impermeable to the vital dye DAPI did not appear to survive long-term environmental pressures any better than isolates of oocysts in which the majority of oocysts were permeable to DAPI. This result is in contrast to that of previous work that showed DAPI− oocysts surviving treatments which killed DAPI+ oocysts (3). Since contact with feces appears to convert DAPI+ oocysts to DAPI−, until further work elucidates the mechanism and significance of this alteration in permeability, rapid dispersal of fecal material is recommended to maximize the vulnerability of oocysts to environmental and disinfectant pressures.

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